REMARKS

The Rejection of Claims 1-10 and 18-39

Claims 1-10 and 18-39 stand finally rejected as anticipated by Drmanac (U.S. 6,667,391). This rejection is respectfully traversed.

To reject a claim as anticipated, each and every element as set forth in the claim must be either expressly or inherently described in a single prior art reference. *Verdegaal Bros. v. Union Oil Co., of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d (BNA) 1051, 1053 (Fed. Cir. 1987). As discussed below, Drmanac fails to teach each element of the claims.

Drmanac allegedly teaches a protein sequence (SEQ ID NO: 23) which shares 42.7% overall sequence identity with the subject application's SEQ ID NO:230. Drmanac further teaches an antibody as "specifically recognizing" or "specific for" a polypeptide of its invention. Col. 67, lines 1-23. Dramanc's use of this term indicates that it has a particular meaning in the art. Nonetheless, the Patent Office takes the position that the term has no meaning in the subject application.

Even if the term had no meaning, and the term in the subject application were very broad, it could not encompass the antibody of Drmanac. Drmanac explicitly teaches that its antibodies exclusively bind to SEQ ID NO: 23.

> Another aspect of the invention is an antibody that specifically binds the polypeptide of the invention....The term "specific for" indicates that the variable regions of the antibodies of the invention recognize and bind polypeptides of the invention exclusively (i.e., able to distinguish the polypeptide of the invention from other similar polypeptides despite sequence identity, homology, or similarity found in the family of polypeptides), but may

> also interact with other proteins (for example, S. aureus protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and in particular, in the constant region of the molecule. Screening assays to determine binding specificity of an antibody of the invention are well known and routinely practiced in the art. For a comprehensive discussion of such assays, see Harlow et al. (Eds), Antibodies A Laboratory Manual; Cold Spring Harbor Laboratory; Cold Spring Harbor, N.Y. (1988), Chapter 6. Col. 66, line 65 to col. 67, line 28.

Antibodies which exclusively bind to SEQ ID NO: 23 cannot bind to a protein of SEQ ID NO: 230, *i.e.,* a different protein. If the Drmanac antibodies cannot bind to a protein of SEQ ID NO: 230, then Drmanac cannot anticipate the present claims, each of which recites the element of specifically binding to an extracellular domain of SEQ ID NO: 230. Since anticipation requires that each and every element of a claim must be met, the failure of Drmanac to fulfill this element prevents Drmanac from anticipating any of the claims.

The Office Action urges that a hypothetical antibody within the scope of the current claims might cross-react with a protein according to Drmanac's SEQ ID NO: 23. Office Action at page 3, lines 10-12. But such a hypothetical antibody is *not* taught by Drmanac. Such a hypothetical antibody within the scope of the current claims would have to bind to an extracellular domain of TEM 17 as shown in SEQ ID NO: 230, as well as cross-react with Drmanac's SEQ ID NO: 23. Such antibodies are explicitly excluded from Drmanac. See U.S. 6,667,391 at col. 67, lines 13-23.

The Patent Office suggests that applicants should provide evidence of a difference between its antibody and the Drmanac antibody. According to the Office Action at page 3, citing

In re Best, such proof is required where a function, property, or characteristic recited is not explicitly disclosed by the reference. See also MPEP §2112 (III). In the present case, however, the Drmanac reference is not silent; Drmanac explicitly discloses that the function, property, or characteristic is not shared by its antibody. Thus, there would be no reason to put applicants to the expense and burden of collecting comparative data, since the reference already provides the information sought.

Specific binding of an antibody is a term of art which ordinarily skilled artisans know. Illustrative of this knowledge are numerous patent claims which recite an antibody that specifically binds to a polypeptide. A selection of these are included in Exhibit 1, including U.S. 7,164,007, U.S. 7,169,565, U.S. 7,166,439, U.S. 7,153,941, and U.S. 7,144,990. These are representative of thousands of such patents which use this terminology. The term "specific binding" clearly has meaning in the art.

The term "specific binding" does have an art-accepted meaning even beyond the patent literature. This art-accepted meaning is evidenced by the FDA's use of the term in describing analyte specific reagents (ASRs). The FDA defines ASRs as "antibodies, both polyclonal and monoclonal,...and similar reagents which, through specific binding or chemical reactions with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens." 21 CFR 864.40 20(a), emphasis added; Exhibit 2.

Additional evidence of such art accepted meaning is found in Fujihara et al. *Infection and Immunity*, 1993:61, 910-918; Exhibit 3. Fujihara characterizes a monoclonal antibody as

specific by direct binding and competitive inhibition assays. "It is intrinsically obvious that the specific binding of antibody or antigen would depend upon both the amount of available antigen and the antibody concentration. Furthermore, specific binding should be saturable under conditions of limited antigen concentration." *Id.* at 914, col. 2, lines 27-31. These examples demonstrate that the term "specific binding" has an art-accepted meaning, so much so that the term is used in the Code of Federal Regulations by an expert agency.

The underlying basis of the rejection is that the Drmanac disclosure of SEQ ID NO:23 inherently discloses antibodies which would specifically bind to isolated areas of identity with SEQ ID NO:230, and therefore "specifically bind" to SEQ ID NO:230. For such a rejection to be proper, specific-binding antibodies to SEQ ID NO:230 must necessarily occur upon practice of the Drmanac reference's teaching.

In replying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art. *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. App. & Inter. 1990). The fact that a certain result or characteristic may occur or be present in the prior art is not sufficient to establish the inherency of that result or characteristic. *In re Rijckaert*, 9 F.3d 1531, 1534, 28 USPQ2d 1955, 1957 (Fed. Cir. 1993). Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient. *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citations omitted).

Because the areas of identity between SEQ ID NO:23 and SEQ ID NO:230 are limited and were not pointed out or taught in the art, antibodies which bind to SEQ ID NO:230 would not necessarily have resulted from Drmanac's teachings. Thus, the rejection is based only on a theory of probabilities and possibilities of generating antibodies which specifically bind to SEQ ID NO:230. Such a rejection is clearly improper.

Withdrawal of this rejection is respectfully requested and a speedy allowance of the claims is sought.

Respectfully submitted,

Dated: February 28, 2007

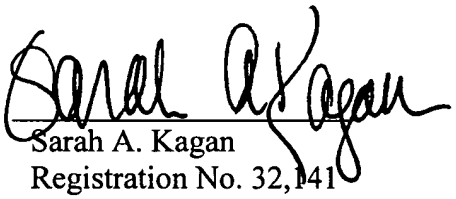
By: 
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EXHIBIT 2

Draft Guidance for Industry and FDA Staff - Commercially Distributed Analyte Specific Reagents (ASRs): Frequently Asked Questions

PDF Printer Version

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.

Document issued on: September 7, 2006

Comments and suggestions regarding this draft document should be submitted within 90 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Alternatively, electronic comments may be submitted to http://www.fda.gov/dockets/ecomments. All comments should be identified with the docket number 2006D-0336.

For questions regarding this document contact Courtney Harper, 240-276-0694 or by email at courtney.harper@fda.hhs.gov

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Devices and Radiological Health
Office of In Vitro Diagnostics Device Evaluation and Safety

Contains Nonbinding Recommendations
Draft - Not for Implementation

Preface

Additional Copies

Additional copies are available from the Internet at: http://www.fda.gov/cdrh/oivd/guidance/1590.pdf . You may also send an e-mail request to dsmica@fda.hhs.gov to receive an electronic copy of the guidance or send a fax request to 240-276-3151 to receive a hard copy. Please use the document number 1590 to identify the guidance you are requesting.

Draft Guidance for Industry and FDA Staff Commercially Distributed Analyte Specific Reagents (ASRs): Frequently Asked Questions

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

INTRODUCTION

This draft guidance document is intended to clarify the regulations regarding commercially distributed analyte specific reagents (ASRs) (21 CFR 809.10(e), 809.30, and 864.4020), and the role and responsibilities of ASR manufacturers. Below we have listed some frequently asked questions and FDA's Office of In Vitro Diagnostic Device Evaluation and Safety's (OIVD's) responses to those questions. Except where otherwise indicated, the use of the term "ASR" in this guidance document refers to commercially distributed ASRs and the term "manufacturer" refers to manufacturers of commercially distributed ASRs.

FDA is providing this guidance in order to eliminate confusion regarding particular marketing practices among ASR manufacturers. As noted in this draft guidance document, ASRs are the building blocks of laboratory-developed tests. (See section II of this guidance.) ASRs are defined and classified in a rule codified at 21 CFR 864.4020. With this draft guidance document, FDA seeks to advise ASR manufacturers that it views the following practices as being inconsistent with the marketing of an ASR, as defined under 21 CFR 864.4020:

- Combining, or promoting for use, a single ASR with another product such as other ASRs, general purpose reagents, controls, laboratory equipment, software, etc.
- Promoting an ASR with specific analytical or clinical performance claims, instructions for use in a particular test, or instructions for validation of a specific test using the ASR.

Some manufacturers have believed that when they combine a Class I ASR, which is exempt from premarket notification requirements under section 510(l) of the Federal Food, Drug, and Cosmetic Act (the Act), 21 U.S.C. 360(l), with other products, or with instructions for use in a specific test, the product remains exempt because of the presence of an ASR. However, as explained in this guidance, when an ASR is marketed in the ways described above, FDA views the product as no longer being an ASR within the meaning of 21 CFR 860.4020 and instead views it as part of a test system.[1]

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

The Least Burdensome Approach

This draft guidance document reflects our careful review of what we believe are the relevant issues related to ASRs and what we believe would be the least burdensome way of addressing these issues. If you have comments on whether there is a less burdensome approach, however, please submit your comments as indicated on the cover of this document.

FREQUENTLY ASKED QUESTIONS

I. The ASR Rule

1. What is the definition of an ASR?

ASRs are defined as "antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reactions with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens." 21 CFR 864.4020(a). ASRs are in vitro diagnostic devices that are regulated by FDA. They are subject to general controls, including the Quality System Regulation (QSR) (21 CFR Part 820), as well as the specific provisions of the ASR regulation (21 CFR 809.10(e), 809.30, 864.4020).

2. What is the ASR rule?

This guidance document refers to three rules as "the ASR rule." The rules, published in 1997, include rules that define and classify ASRs (21 CFR 864.4020), impose restrictions on the sale, distribution, and use of ASRs (21 CFR 809.30), and establish requirements for ASR labeling (21 CFR 809.10(e)).

3. What was the objective of the ASR rule?

The ASR rule was designed to accomplish several policy objectives. These include ensuring the quality of materials used as components of in-house laboratory tests, and providing appropriate labeling so that healthcare users would understand how these tests were being validated. 62 FR 62244. FDA adopted the approach of regulating most ASRs using general controls and exempting them from premarket notification requirements as the least burdensome approach. This approach relies primarily on current Good Manufacturing Practices (cGMPs), medical device reporting, and labeling requirements, along with the Clinical Laboratory Improvement Amendments (CLIA), 42 U.S.C. 263a, to adequately control the risks associated with these devices. 62 FR 62252.

4. What does the ASR rule require?

The rule classifies most ASRs as Class I devices subject to general controls under section 513 (a)(1)(A) of the Act. The general controls require ASR manufacturers to register and list their devices, 21 CFR 807.20(a), submit medical device reports (21 CFR Part 803), follow labeling requirements, 21 CFR 809.10(e), and follow cGMPs, 21 CFR 809.20(b). The rule also restricts the sale, use, distribution, labeling, advertising and promotion of ASRs. 21 CFR 809.30. One of these restrictions allows only physicians and other persons authorized by applicable State law

to order in-house tests that are developed using ASRs. 21 CFR 809.30(f). Another restriction requires the laboratory that develops an in-house test using an ASR to add a statement disclosing that the laboratory developed the test and it has not been cleared or approved by FDA when reporting the test result to the practitioner. 21 CFR 809.30(e).

The restrictions also prohibit advertising and promotional materials for ASRs from making any claims for clinical or analytical performance. 21 CFR 809.30(d)(4). Consistent with this restriction, the labeling for Class I, exempt ASRs must bear the statement, "Analyte Specific Reagent. Analytical and performance characteristics are not established." 21 CFR 809.10(e)(1)(x). Manufacturers who wish to make analytical and/or clinical performance claims for a product should submit an application to FDA for premarket review rather than marketing the product as an ASR. For example, performance claims might include statements such as, "This ASR can be used to quickly and accurately detect [a mutation] associated with [a disease]."

In addition, the rule classifies certain ASRs as Class II or III devices that are subject to premarket notification or premarket approval application requirements in addition to the general controls described above.

5. Are some ASRs Class II or Class III, requiring a premarket submission?

Yes. Although most ASRs are Class I, there are some ASRs that are Class II and Class III and that must be cleared or approved by FDA before they can be marketed in the United States. 21 CFR 864.4020. FDA classifies medical devices, including diagnostic devices such as ASRs, into Class I, II, or III according to the level of regulatory control that is necessary to provide a reasonable assurance of safety and effectiveness. These classifications include consideration of the level of risk associated with the device. 21 U.S.C. 360c. The classification of an ASR determines the appropriate premarket process.

An ASR is a Class II device if the reagent is used as a component in a blood banking test of a type that has been classified as a Class II device (e.g., certain cytomegalovirus serological and treponema pallidum nontreponemal test reagents). 21 CFR 864.4020(b)(2).

An ASR is a Class III device if the reagent is intended as a component in tests intended either:

- to diagnose a contagious condition that is highly likely to result in a fatal outcome and prompt, accurate diagnosis offers the opportunity to mitigate the public health impact of the condition (e.g., human immunodeficiency virus (HIV/AIDS) or tuberculosis (TB)); or
- for use in donor screening for conditions for which FDA has recommended or required testing in order to safeguard the blood supply or establish the safe use of blood and blood products (e.g., tests for hepatitis or for identifying blood groups). 21 CFR 864.4020(b)(3).

6. How does a manufacturer know whether its device is an ASR?

We recommend that ASR manufacturers consult this document for guidance on whether their product is or is not within the scope of the ASR rule. Manufacturers should contact FDA if they are unsure about the classification of their device to discuss any applicable regulatory requirements. Manufacturers who wish to obtain FDA advice on this matter in advance of marketing may consult with OIVD.

II. What Meets the ASR Definition?

The ASR definition has given rise to confusion about which products fall within and outside of its bounds. Some of this confusion arises because a product that might in some circumstances be an ASR, will no longer be one due to its marketing and claims.

In the preamble to the ASR rule, FDA stated that ASRs may be thought of as the "active ingredients" of tests that are used to identify one specific disease or condition. ASRs are purchased by manufacturers who use them as components of tests that have been cleared or approved by FDA and also by clinical laboratories that use the ASRs to develop in-house tests used exclusively by that laboratory. 62 FR 62243, 62244. This is in contrast with what the preamble referred to as a "kit or system for 'in vitro diagnostic use'" that has a proposed intended use, indications for use, instructions for use, and performance characteristics. 62 FR 62243, 62250. FDA designed the ASR rule to require that ASR manufacturers take certain actions, such as following cGMPs, to help ensure the safety and effectiveness of their devices. A premise underlying the rule, however, is that laboratories, rather than ASR manufacturers, develop the test in which the ASR is used and provide all necessary verification and validation.

Based upon this description, together with the ASR definition, FDA views an ASR as having the following characteristics:

- a single moiety;
- a single endpoint;
- no instructions or performance claims; and
- not promoted for use on specific instruments or in specific tests or test systems.

7. What are some examples of entities that FDA considers to be ASRs?

Examples of molecules that are ASRs are a single antibody (e.g., an anti-troponin I antibody), a single nucleotide primer (e.g., a forward primer for amplification of the ΔF508 locus of the gene encoding the cystic fibrosis transmembrane regulator (CFTR)), and a single purified protein or peptide (e.g., purified estrogen receptor protein or purified B-type natriuretic peptide). The above-listed examples would not be considered ASRs if they are marketed with clinical or analytical performance claims (e.g., cystic fibrosis genotyping, identification of cardiac risk).

8. What are some examples of entities that FDA does not consider to be ASRs?

- Multiple moieties (e.g., antibodies, probes, primers) bundled together in a pre-configured or optimized manner so that they are intended to identify and quantify more than one chemical substance or ligand. Such products are not ASRs because ASRs are defined as intended for use in "identification and quantification of an *individual* chemical substance or ligand in biological specimens." 21 CFR 864.4020(a) (emphasis added). As a result, FDA considers such products to be test systems, rather than ASRs. This means that products that might be ASRs when marketed individually, would not be considered ASRs when combined or multiplexed because they are no longer intended to identify an "individual chemical substance." ASRs are intended to be individual building blocks of tests that a laboratory develops. When a manufacturer combines ASRs, it has taken steps to build a particular test.
- Test systems. FDA considers a product a test system rather than an ASR when it

includes more than a single ASR (i.e., it includes some or all of the products needed to conduct a particular test such as more than one ASR, general reagents, controls, equipment, software, etc.) and/or has instructions for use.
- Control material.
- Products that have specific performance claims, or procedural instructions, or interpretations for use.
- Reagents that are extensively processed (e.g., arrayed on beads). This type of modification is an optimization of the reagent to create a particular intended use that is more specific than the broad intended use described in the ASR definition. 21 CFR 864.4020(a).
- Reagents offered with software for interpretation of results.
- Products that do not meet the ASR definition, such as software for interpretation of assay results, or microarrays.

9. How do General Purpose Reagents compare to ASRs?

A General Purpose Reagent (GPR) is "a chemical reagent that has general laboratory application, that is used to collect, prepare, and examine specimens from the human body for diagnostic purposes, and that is not labeled or otherwise intended for a specific diagnostic application." 21 CFR 864.4010(a). Like ASRs, GPRs are not labeled for a specific clinical or diagnostic use. Because GPRs are not analyte-specific, they should be able to be combined with, or used in conjunction with more than one ASR. In contrast, as stated above, an ASR is a specific chemical component, probe, or antibody that can detect an individual chemical substance or ligand. An ASR is considered the "active ingredient" or "building block" of a laboratory-developed test.

III. Manufacturer Marketing Practices

10. To whom can manufacturers sell ASRs?

ASRs may only be sold to:

- in vitro diagnostic manufacturers;
- clinical laboratories regulated under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), as qualified to perform high complexity testing or clinical laboratories regulated under VHA Directive 1106; and
- organizations that use the reagents to make tests for purposes other than providing diagnostic information to patients and practitioners, e.g., forensic, academic, research and other nonclinical laboratories.
  21 CFR 809.30 (b).

11. Can a manufacturer or distributor promote specific ASRs and GPRs for use together in developing a test?

No. As explained above, ASRs are considered individual "building blocks" of laboratory tests and GPRs may not be intended for a specific diagnostic application. 21 CFR 864.4010, 864.4020. Although a GPR may consist of a single substance or a formulation of multiple substances as defined by 21 CFR 864.4010, it should be intended for general rather than specific use. A product that is promoted for use with an ASR is intended for a specific diagnostic use with that ASR and therefore would not meet the GPR definition of being for

"general laboratory application." Similarly, a product that is promoted for use with a specific GPR is intended for a particular intended use rather than as an ASR, which is a building block of laboratory-developed tests. Therefore, a manufacturer who wishes to market its products as GPRs or ASRs should not promote or sell them together, including in test system configurations.

We recommend that manufacturers who wish to market products as ASRs, rather than as test systems, avoid listing ASRs, GPRs, and/or controls in catalogues, web sites, and other promotional materials, in small groupings that suggest these devices should be used together for a specific purpose. For example, primers specific for Factor V, Factor II, and MTHFR listed together with complementary probes and control material suggest that this group of reagents may be used together as a type of thrombophilia panel. Manufacturers who wish to promote products together in this manner should submit a PMA or 510(k) to FDA for approval or clearance of a test system.

To avoid promoting products as test systems rather than as separate ASRs and GPRs, we recommend that manufacturers list ASRs and GPRs in a fashion that is not associated with use in a particular test (e.g., alphabetically, or by reagent type [primers together, buffers together]).

12. Can the manufacturer include instructions with an ASR?

ASR manufacturers should not provide such instructions with an ASR. As explained above, FDA views reagents that are sold with instructions for developing or performing a test as test systems rather than as ASRs because an ASR is a building block for laboratory-developed tests and has no analytical or clinical performance claims. 21 CFR 809.10(e)(1)(x), 809.30(d)(4). Instructions for use of an ASR in a particular test constitute a claim that, when used as directed, the ASR will perform to detect a particular chemical substance or ligand.

On the other hand, instructions for storage and handling of an ASR must be provided. 21 CFR 809.10(e)(1)(vi). In addition, scientific information may be included on chemical/molecular composition, nucleic acid sequence, binding affinity, cross-reactivities, known mutations associated with the sequence, and interaction with substances of known clinical significance. 21 CFR 809.10(e)(1)(iv).

13. Can a manufacturer or distributor tell a laboratory which ASRs are useful for a particular application, for example, which monoclonal antibodies or probes are useful for leukemia or lymphoma testing?

Manufacturers and distributors should not make claims to physicians or laboratories regarding analytical or clinical performance for ASRs. The laboratories, not the manufacturers or distributors, should be responsible for the performance of the test. 21 CFR 809.30(d)(4). In addition, manufacturers should take care to avoid names for their ASRs that describe a specific clinical use.

ASR labeling may indicate the affinity of the reagent, such as "anti-estrogen receptor antibody" or "ΔF508 CFTR nucleic acid probe." Other similar information, such as the affinity, target, or sequence of a DNA probe or a protein sequence, may also be provided because it describes what the ASR is specific for but does not claim to produce a particular clinical or analytical result. But a name such as "Cystic Fibrosis ASR" describes a specific clinical use for the

product and FDA, therefore, would not consider such a product to be an ASR.

14. Can an ASR manufacturer supply quality control materials/reagents that can be used with an ASR?

Yes, but these materials should be promoted independently of specific ASRs. Marketing such materials with an ASR may make it appear that the manufacturer is actually marketing a test system, which could trigger premarket review requirements. Quality control materials should be promoted and sold using existing FDA classifications for quality control material. (e.g., 21 CFR 862.1660, 862.3280, 864.8625)

15. Can a manufacturer or distributor market software for use with an ASR?

If a manufacturer chooses to market software for use with its product, then the products together would be considered a test system, rather than an ASR. ASR manufacturers should not promote or sell software for use with a particular ASR.

16. What types of instrumentation can manufacturers promote for use with laboratory-developed tests?

Manufacturers should not promote closed system laboratory instruments (i.e., when the user does not have the ability to modify instrument settings, or the design of the instrument allows only a specific proprietary reagent technology or assay method to be used) for use in conjunction with particular ASRs. FDA would consider promotion of such instrumentation with a specific ASR to be promotion of a test system.

In contrast, open system instruments that have user-defined capabilities, which allow the user to define, optimize, and validate the test performance characteristics and interpretation criteria, may be promoted for use generally in laboratory-developed tests. Examples of these instruments include spectrophotometers, HPLC, and flow cytometers. The instrumentation should be able to run various assays, allow parameter modification, or have a user interface that the laboratory can modify to define specific parameters for their laboratory-developed assay. If instrumentation is used, the laboratory should select the instrumentation and validate the performance of the laboratory-developed assay on that instrument.

17. Can the ASR manufacturer help with the verification of performance specifications of a test that utilizes its ASR?

Under the CLIA regulations, the laboratory must conduct verification of performance specifications. 42 CFR 493.1213. This validation by the laboratory is the minimum required under CLIA for the laboratory to generate clinical results for tests of high complexity. If a manufacturer or distributor wishes to market its product as an ASR, it should not assist with the validation of a laboratory-developed test using its specific ASR.

18. What type of information about a particular ASR can an ASR manufacturer provide to a laboratory?

A manufacturer may provide laboratories with information, including peer-reviewed and published/presented literature, limited to characteristics of the ASR itself. If a manufacturer intends to market its products as ASRs, it should not promote its products with literature and

other materials that include information on use of the ASR in a specific test or test system. This would include information regarding an ASR's clinical utility and clinical performance as well as specific instructions-for-use and validation protocols. FDA views this type of information, when provided by the manufacturer, as evidence of intent to market a test system with instructions for use and validation. FDA would not view a product that is promoted with such information as an ASR.

IV. Research and Investigational Use of ASRs

19. Can ASRs be used for research?

Yes, ASRs can be used for research applications. The ASR requirements, including the need for the laboratory report disclaimer, apply only to clinical diagnostic use of these products and not to research applications. 21 CFR 864.4020(a)(2).

20. How is the ASR rule related to in vitro diagnostic products labeled for research or investigational use?

Products labeled for research use only (RUO) or investigational use only (IUO) are IVDs in different stages of development.

- FDA considers RUO products to be products that are in the laboratory research phase of development, that is, either basic research or the initial search for potential clinical utility, and not represented as an effective in vitro diagnostic product. During this phase, the focus of manufacturer-initiated studies is typically to evaluate limited-scale performance and potential clinical or informational usefulness of the test. These products must be labeled "For Research Use Only. Not for use in diagnostic procedures." as required under 21 CFR 809.10 (c)(2)(i).
- FDA considers IUO products to be products that are in the clinical investigation phase of development. They may be exempt from the investigational device (IDE) requirements of 21 CFR Part 812 (21 CFR 812.2(c)), or may be regulated under 21 CFR Part 812 as either a non-significant risk device or a significant risk device. Diagnostic devices exempt from IDE requirements cannot be used for human clinical diagnosis unless the diagnosis is being confirmed by another, medically-established diagnostic product or procedure (21 CFR 812.2(c)(3)(iv)). During this phase, the safety and effectiveness of the product are being studied; i.e., the clinical performance characteristics and expected values are being determined in the intended patient population(s). These products must be labeled, "For Investigational Use Only. The performance characteristics of this product have not been established." 21 CFR 809.10(c)(2)(ii).

21. What is the difference in GMP requirements for manufacturers of an ASR versus an RUO reagent?

Manufacturers establish and follow cGMPs, as established in the quality system regulation, to help ensure that their products are manufactured under controlled conditions that assure the devices meet consistent specifications across lots and over time. ASRs must be manufactured following cGMPs. 21 CFR 809.20. FDA does not expect RUO reagents to be manufactured in compliance with cGMPs because products labeled as RUO reagents cannot be used as clinical diagnostic products. 21 CFR 809.10(c)(2)(i).

[1] FDA's use of the term "test system" in this guidance document is not linked to definitions in 42 CFR Part 493. See question 8 for discussion of what FDA considers a "test system."

Updated September 5, 2006

EXHIBIT 3

INFECTION AND IMMUNITY, Mar. 1993, p. 910–918
0019-9567/93/030910-09$02.00/0

Vol. 61, No. 3

Characterization of Specific Binding of a Human Immunoglobulin M Monoclonal Antibody to Lipopolysaccharide and Its Lipid A Domain

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The human immunoglobulin M monoclonal antibody HA-1A was first described as an antibody which bound specifically to the lipid A region of lipopolysaccharide (LPS) (N. N. H. Teng, H. S. Kaplan, J. M. Herbert, C. Moore, H. Douglas, A. Wunderlich, and A. Braude, Proc. Natl. Acad. Sci. USA 82:1790–1794, 1985) and provided significant protection when administered to patients with gram-negative bacteremia and shock (E. J. Ziegler, C. J. Fisher, Jr., C. L. Sprung, R. C. Straube, J. C. Sadoff, G. E. Foulke, C. H. Wortel, M. P. Fink, R. P. Dellinger, N. N. H. Teng, I. E. Allen, H. J. Berger, G. L. Knatterud, A. F. LoBuglio, C. R. Smith, and the HA-1A Sepsis Study Group, New Engl. J. Med. 324:429–436, 1992). Since that original report, questions have arisen in the scientific literature concerning the specificity of this antibody in LPS and/or lipid A binding. Experiments have, therefore, been carried out with a variety of assay formats to determine the capacity of this HA-1A antibody to bind to lipid A and LPS. Direct binding experiments with a sensitive enzyme-linked immunosorbent assay (ELISA) system have established that HA-1A will bind to purified lipid A from both *Escherichia coli* and *Salmonella* spp. These results have been confirmed by using a fluid-phase antigen-antibody competitive inhibition assay with purified lipid A and an antibody-antibody competitive inhibition assay with a monoclonal antibody with known specificity for lipid A. The HA-1A monoclonal antibody has also been shown to bind to a panel of R-chemotype LPS by ELISA and, unlike many other previously reported anti-lipid A antibodies, binding of HA-1A to R-chemotype LPS and lipid A is comparable. Although binding of HA-1A to S-LPS (smooth, wild-type LPS) could not be detected by direct ELISA, competitive inhibition experiments with some preparations of S-LPS have been able to show specific HA-1A binding. Collectively, these data confirm the binding specificity of HA-1A for the lipid A component of LPS and provide evidence that this monoclonal antibody manifests a relatively uncommon profile in its capacity to bind lipid A and R-chemotype LPS as well as some preparations of S-LPS.

There exists an abundance of experimental evidence to support the concept that bacterial endotoxic lipopolysaccharide (LPS) derived from the outer cell membrane of gram-negative bacteria is a significant contributing factor to the pathogenesis of septic shock (20). In part, such evidence has been derived from studies in which highly purified LPS administered to human volunteers has been demonstrated to reproduce many of the pathophysiological manifestations of gram-negative bacteremia and shock (6). Research during the past decade has, in fact, established that cytokines and other inflammatory mediators released from host inflammatory mediator cells in response to LPS are primarily responsible for the vascular pathology and resultant multisystem organ failure characteristic of this disease (19).

Among the most significant advances in our understanding of endotoxemia has been the elucidation of the chemical structure of LPS and its biologically active lipid A component. In this respect, it is now generally recognized that most LPS from gram-negative organisms contain antigenically diverse polysaccharide structures termed O antigen polysaccharide, as well as chemically conserved domains termed inner-core oligosaccharide and lipid A. The recent demonstration that lipid A, prepared by total organic synthesis, manifests endotoxic properties indistinguishable from those of bacterially derived lipid A has provided unequivocal proof of the importance of this structure to the biological activity of LPS (reviewed in reference 14).

In spite of a vast increase in knowledge of the chemistry and biology of endotoxin and its contribution to gram-negative infectious disease, efforts to reduce morbidity and mortality in septic patients with gram-negative bacteremia have, in general, met with only limited success (4, 15, 30, 31). In experimental animals, however, specific antibody directed against O antigen polysaccharide has repeatedly been shown to provide impressive levels of protection during challenge with the homologous LPS (reviewed in reference 24). However, because the array of unique O antigen polysaccharides likely to be encountered in endotoxemic patients would be large, the use of O antigen-specific immunologic reagents in the treatment of septic patients has not been considered practical. As an alternative, investigators during the past several decades have pursued the development of immunologic reagents with antigenic specificity for shared determinants on the LPS macromolecule, specifically lipid A and core oligosaccharides. Early successes in a variety of experimental models of endotoxemia provided a rational basis for a major clinical trial with antiserum from human volunteers previously immunized with a gram-negative organism (J5) lacking O antigen polysaccharide structural determinants (31). The success of this antiserum in reducing mortality in patients with gram-negative bacteremia and shock clearly established that antiserum to LPS may be

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an effective immunotherapy in the treatment of such patients.

In 1985, Teng et al. (27) reported the development of a human monoclonal antibody (MAb) with antigenic specificity for the lipid A component of LPS. Published studies by these investigators suggested that this MAb, termed HA-1A, would bind to both LPS and to the lipid A purified from the LPS. These results suggested that this MAb was, in fact, specific for binding to lipid A. A well-designed, double-blind, placebo-controlled, clinical trial with HA-1A MAb has recently been completed and has established that this antibody is also efficacious in reducing the mortality of patients with gram-negative bacteremia and shock (30). Of significance, the protective efficacy of HA-1A was comparable to that seen in the earlier J5 polyclonal antibody clinical trial (31). Collectively, these two studies suggest that antibody to LPS and lipid A may be of value in the treatment of patients with gram-negative bacteremia, particularly those with septic shock.

Recently, concerns have been raised in the scientific literature as to the validity of the conclusion that MAb HA-1A is, in fact, specific for the lipid A domain of LPS (2, 7). Such concerns have derived from immunoassay systems in which this MAb has been reported to bind to a wide variety of bacterial antigens (1). However, given that immunoassays designed to assess specific binding to lipid A may present difficulties not encountered with many antigens (5, 10, 25), it is important that considerable attention be devoted to the establishment of appropriate assay conditions. In this article, we report the development of an indirect enzyme-linked immunosorbent assay (ELISA) for the determination of specific binding of MAb HA-1A compared with a previously reported MAb with reported specificity for lipid A. Our results confirm that this antibody reacts specifically with lipid A and LPS.

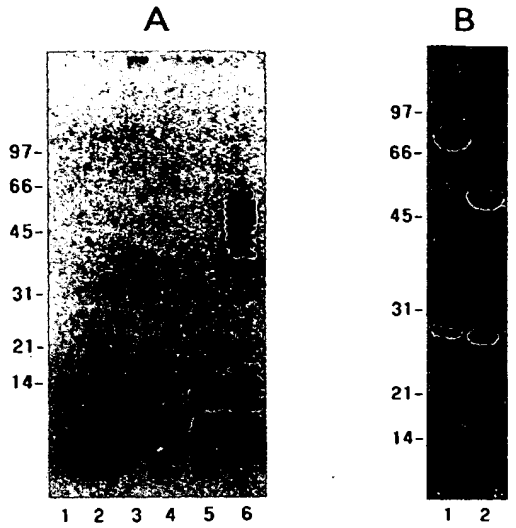

FIG. 1. SDS-PAGE analysis of LPS preparations and monophosphoryl lipid A (A) and MAbs (B). (A) Results of silver stain of LPS fractionated by 8 to 20% gradient SDS-PAGE. Lanes: 1, 5 μg of monophosphoryl lipid A from *S. minnesota* R595; 2, 2.5 μg of Re-LPS from *S. minnesota* R595; 3, 2.5 μg of Rd_1-LPS from *S. minnesota*; 4, 2.5 μg of Rc-LPS from *E. coli* J5; 5, 2.5 μg of Ra-LPS from *S. minnesota* R60; 6, 5 μg of S-LPS from *E. coli* O111:B4. The electrophoresis was performed at constant current (3 mA) for 18 h at room temperature. (B) Results of Coomassie blue stain of MAbs electrophoresed on SDS-PAGE (11% polyacrylamide). Lanes: 1, 15 μg of MAb HA-1A; 2, 15 μg of MAb 8A1. The electrophoresis was performed at constant current (20 mA) for 4 h at 4°C.

MATERIALS AND METHODS

LPS and lipid A. The S-LPS (smooth, wild-type LPS) from *Escherichia coli* O111:B4 was extracted and purified from late-log-phase bacteria by the method of Westphal and Jann (29) as modified by Morrison and Leive (18). The deep rough chemotype LPS from *Salmonella minnesota* R595 (Re-LPS) and *S. minnesota* R7 (Rd_1-LPS) were extracted in our laboratory by the phenol-chloroform-petroleum ether method exactly as described by Galanos et al. (12). The Rc-LPS from *E. coli* J5, the Ra-LPS from *S. minnesota* R60, and monophosphoryl lipid A from *S. minnesota* R595 were purchased from List Laboratories (Campbell, Calif.). Lipid A from *E. coli* J5 was prepared by mild acid hydrolysis also as previously described (21). Unless otherwise indicated, all experiments described in the text were carried out with the *S. minnesota* R595 monophosphoryl lipid A preparation. All LPS prepared in our laboratory were free of A_{260} and by chemical estimation of protein contamination were less than 1% by weight. LPS and lipid A preparations obtained from List Laboratories were reported by the manufacturer to be of equivalent degrees of purity. A representative profile of the LPS preparations used in these studies after silver-stain sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Tsai and Frasch (28) is shown in Fig. 1A.

Antibody preparations. The HA-1A human immunoglobulin M (IgM) MAb described by Teng et al. (27) and the 8A1 mouse IgG, anti-lipid A antibody described by Bogard et al. (3) were provided by Centocor Inc. (Malvern, Pa.) as stock solutions in pyrogen-free phosphate-buffered saline (PBS) at a concentration of 5 to 10 mg/ml. The purity of these MAbs was assessed by SDS-PAGE as described by Laemmli (17) followed by staining with Coomassie blue. Molecular weight markers were purchased from Bio-Rad Inc. (Richmond, Calif.). The SDS-PAGE profiles of these antibodies are shown in Fig. 1B and confirm the purity of these reagents. Peroxidase-conjugated goat anti-human IgM (μ-chain specific) and goat anti-mouse IgG (Fc-specific) were purchased from Sigma Chemicals (St. Louis, Mo.). A control mouse IgG2a MAb specific for *E. coli* O111:B4 LPS O antigen (9) was also provided by Centocor Inc. At the concentrations used in the experiments described, these conjugated antibodies manifest no detectable cross-reactivity with mouse IgG or human IgM.

ELISAs. Extensive preliminary experiments were carried out to determine optimal conditions of LPS or lipid A binding and blocking conditions to minimize nonspecific antibody binding. These included different methodologies for antigen binding (time, temperature, concentration, LPS solubilization, microtiter plate source) and blocking reagents (albumin, gelatin, skim milk, fetal calf serum). For all of the experiments described, flat-bottom 96-well polystyrene microtiter plates (Immulon 1; Dynatech Laboratories, Alexandria, Va.) were pretreated with 150 μl of 25-μg/ml poly-*l*-lysine (Sigma Chemicals) for 30 min at room temperature, and then the poly-*l*-lysine solution was removed as described previously (13). Lyophilized LPS, *E. coli* lipid A, or monophosphoryl *S. minnesota* lipid A was reconstituted to 1.0 to 5.0 mg/ml in pyrogen-free distilled water, sonicated for 3 min at maximum output on a Heat-Systems Ultrasonics Sonicator (Farmingdale, N.Y.), and diluted to 50 μg/ml with PBS

(pH 7.4). Then, 50-μl volumes of serial twofold dilutions of LPS or lipid A in PBS were added to individual microtiter wells with concentrations ranging from 50 to 1.6 μg/ml, with additional wells receiving PBS alone as no-antigen controls. Plates were incubated at 37°C for 1 h for LPS antigen and at 4°C for 18 h for lipid A antigen. After incubation, excess unbound antigen was removed and the wells were washed three times with PBS. Residual nonspecific binding was blocked by the addition of 100 μl of RPMI 1640 tissue culture medium containing 10% fetal bovine serum (JRH Biosciences, Lenexa, Kans.) for 2 h at 37°C. The blocking agent was then removed, and the individual wells were washed an additional three times with PBS containing 0.05% Tween 20 (PBS-Tween; Sigma Chemicals).

Various concentrations of anti-lipid A antibody (HA-1A or 8A1) or anti-LPS antibody (5B10) diluted in PBS (pH 7.0 containing 0.1 M NaCl) were then added in a volume of 50 μl, and binding was carried out at 37°C for 2 h. Wells were then washed three times with PBS-Tween and specific peroxidase-conjugated second antibodies diluted in PBS-Tween added in volumes of 50 μl for a period of 30 min at 37°C. Dilutions of antibody were approximately 1:5,000 for the anti-human IgM and 1:2,000 for the anti-mouse IgG. Wells were again washed three times with PBS-Tween. Bound peroxidase was detected by the addition of 50 μl of 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Labs, Gaithersburg, Md.) for approximately 10 min, followed by addition of 50 μl of 1.0 M H_3PO_4. Absorbance was determined on a Titertek Multiskan spectrophotometer (Flow Laboratories, McLean, Va.) at a wavelength of 450 nm.

Competitive inhibition assays. For solid-phase antibody-antibody competitive inhibition experiments in which the influence of one anti-lipid A antibody on the binding of a second anti-lipid A antibody was assessed, both antibodies were added simultaneously to antigen-coated microtiter plates. For fluid-phase antigen-antibody competitive inhibition experiments, antibody was incubated with competing antigen in microcentrifuge tubes for 2 h at room temperature and then centrifuged at 9,000 × *g* for 10 min. Aliquots of supernatants were then assayed for residual solid-phase antigen binding.

RESULTS

Binding of MAbs HA-1A and 8A1 to lipid A. Both HA-1A and 8A1 have been reported to have binding specificity for the isolated lipid A component of LPS (3, 27). To assess this binding specificity with the ELISA system developed in our laboratory and to minimize potential contributions of nonspecific binding, the capacity of these antibodies to bind lipid A was assessed both at different antigen (lipid A) dilutions (on the solid phase) and also at different antibody concentrations. A summary of the results of three sets of experiments for the HA-1A and 8A1 MAbs is shown in Fig. 2a and b, respectively. These results indicate that binding of both antibodies is increased in the presence of either increasing concentrations of lipid A or increasing concentrations of antibody. Virtually identical results were obtained with *E. coli* lipid A (data not shown). When the data shown in Fig. 2 are analyzed for binding as a function of the amount of antibody at a fixed amount of lipid A, it is clear that binding of both antibodies is saturable at absorbance values below the maximal linearity range (approximately 1.6) of the spectrophotometer (Fig. 2c and d). A comparison of the binding profiles as a function of antibody concentration suggests that the relative affinity of 8A1 for lipid A is greater than that of HA-1A. Comprehensive experiments carried out with control IgM antibody (both monoclonal and polyclonal) have not demonstrated significant levels of nonspecific binding to lipid A under these conditions (data not shown).

Because both the HA-1A and 8A1 MAbs manifest binding specificity for lipid A, it was of interest to investigate whether these two antibodies might detect similar epitopes. Competitive inhibition studies were therefore carried out to assess whether 8A1 would inhibit the binding of HA-1A to lipid A. Increasing concentrations of either 8A1 or a control antibody directed against O antigen specific oligosaccharide of *E. coli* O111:B4 LPS (5B10) were added to a constant amount of HA-1A, and the mixtures were then analyzed for specific HA-1A binding to lipid A. The results of one such experiment in which both the lipid A and HA-1A concentrations varied are shown in Fig. 3a for 8A1 and 3b for 5B10. The dose dependence of inhibition at a fixed concentration of lipid A (50 μg/ml) is shown in Fig. 3c. These data confirm the dose-dependent inhibition of HA-1A binding by 8A1 but not 5B10 and are consistent with, but not conclusive evidence of, a shared epitope for both HA-1A and 8A1 binding to lipid A.

As an alternative method to confirm HA-1A binding to lipid A, soluble antigen competitive inhibition assays were also carried out with lipid A. In these experiments, increasing amounts of lipid A were preincubated for 2 h at 23°C with HA-1A and then centrifuged for 10 min at 9,000 × *g*. (We have found that the centrifugation step is necessary because the immune complexes of HA-1A and lipid A appear to have increased affinity to either bind nonspecifically to microtiter plates or specifically via unoccupied antigen combining sites in the IgM-containing immune complexes.) Supernatants were then assayed for residual binding to immobilized lipid A. Representative results of one such experiment are shown in Fig. 4 and confirm that soluble lipid A is an efficient and effective inhibitor of HA-1A binding to immobilized lipid A. These results would also support the conclusion that binding of HA-1A to immobilized lipid A is not a reflection of the generation of unique neoantigens created by absorption of lipid A to the microtiter plate.

Binding of MAb HA-1A and 8A1 to R-chemotype LPS. In order for an anti-lipid A MAb to be effective in the treatment of endotoxic shock, it is necessary that such an antibody also be capable of binding to lipid A in association with polysaccharide. We have used a similar ELISA format to investigate the relative capacities of HA-1A and 8A1 to bind to a panel of R-chemotype LPS. As described earlier, we have determined antibody binding both as a function of antigen (R-chemotype LPS) used to coat the microtiter plate and as a function of antibody (HA-1A and 8A1) concentration. The results of the binding of HA-1A and 8A1 to Re-, Rd_1-, Rc-, and Ra-LPS are summarized in Fig. 5a and 5b, respectively. These data demonstrate that HA-1A can effectively bind to all of the R-chemotype LPS tested. In contrast, the 8A1 MAb manifests a marked preference for binding to R-chemotype LPS with lower amounts of core oligosaccharide (i.e., Re-LPS), and binding is significantly reduced when Rc- and Ra-LPS are used as antigens.

In order to assess the relative efficacy of binding of HA-1A and 8A1 to the various LPS chemotypes, we have analyzed the results of these binding studies (and those shown in Fig. 2a and b) at a constant LPS or lipid A concentration (25 μg/ml) as a function of MAb concentration. The results of this analysis for both HA-1A and 8A1 binding are shown in Fig. 6a and b, respectively. As suggested above, the 8A1 MAb clearly manifests a significant preference for binding to

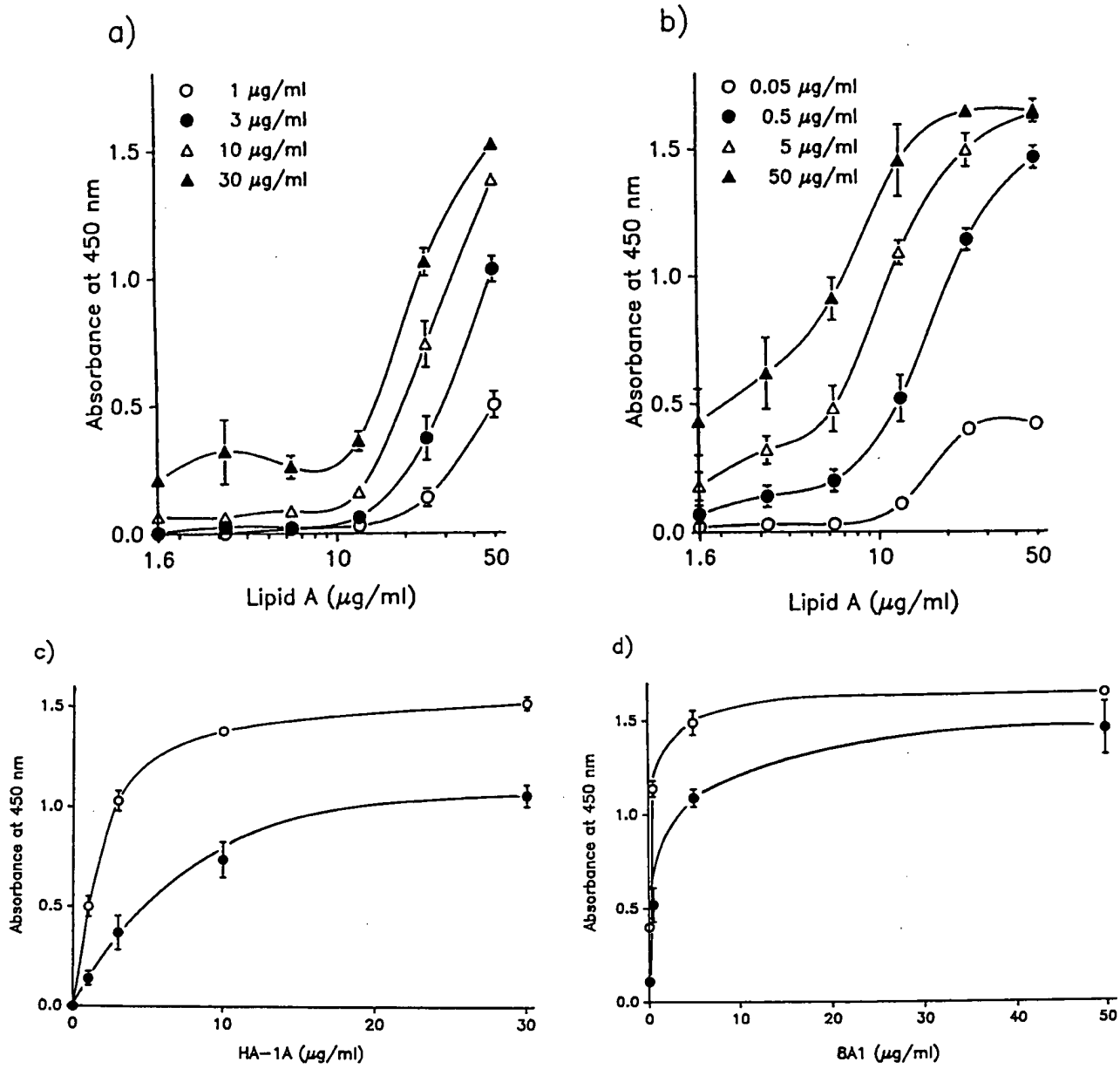

FIG. 2. Comparison of the binding of HA-1A (a) and 8A1 (b) to lipid A in the ELISA. The 96-well microtiter plates were coated with twofold serial dilutions of lipid A as described in Materials and Methods. HA-1A and 8A1 were diluted in PBS (pH 7.0) containing 0.1 M NaCl at 1, 3, 10, and 30 μg of HA-1A per ml or 0.05, 0.5, 5, and 50 μg of 8A1 per ml and then were added to the wells. The data are analyzed for binding as a function of the antibody concentration at a fixed amount of lipid A (25 [●] or 50 [O] μg/ml for HA-1A [c]; 12.5 [●] or 25 [O] μg/ml for 8A1 [d]). Data points represent the means ± standard errors of triplicate determinations.

isolated lipid A and Re-LPS, with progressively reduced binding to R-chemotype LPS containing increasing amounts of core oligosaccharide. Relative binding of 8A1 to Ra-LPS is reduced almost four orders of magnitude compared with lipid A binding. In marked contrast to these results, the HA-1A binding profiles vary less than threefold in terms of increase or decrease between the lipid A profile and any of the R-chemotype LPS binding profiles. Because rigorous efforts to precisely quantitate the amount of the various antigen preparations deposited on the microtiter plates have not been attempted, it is not possible to attribute any significance to the minor differences observed with HA-1A binding in Fig. 6a. In any case, these data establish that, in contrast to 8A1, HA-1A manifests a relatively broad spectrum of LPS binding to the R-chemotype LPS.

Binding of MAb HA-1A to S-LPS. A final set of experiments was carried out to assess the capacity of HA-1A to bind to O antigen containing S-LPS. Of interest, when the ELISA format described above was used, experiments have been uniformly unsuccessful in detecting any binding to *E.*

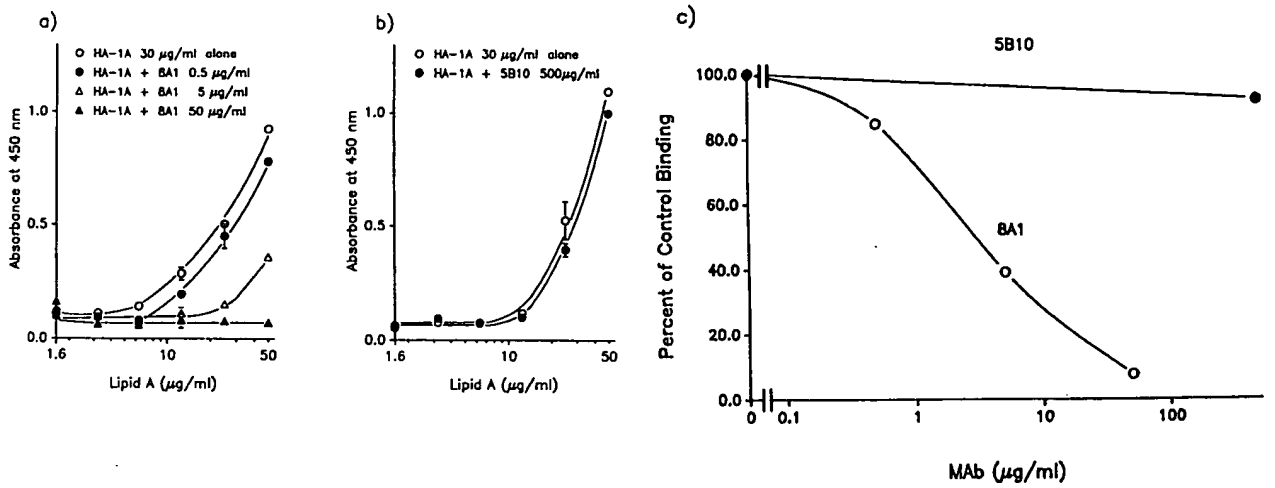

FIG. 3. Competitive inhibition of HA-1A binding to immobilized lipid A by 8A1 and 5B10. The 96-well microtiter plates were coated with twofold serial dilutions of lipid A as described in Materials and Methods. (a) HA-1A (30 μg/ml, in PBS, pH 7.0, containing 0.1 M NaCl) and 8A1 (0, 0.5, 5, and 50 μg/ml, in PBS, pH 7.0, containing 0.1 M NaCl) were mixed in test tubes and then immediately added to the wells. (b) Effects of MAb 5B10 (500 μg/ml) on HA-1A (30 μg/ml) binding were tested in the same way as in panel a. (c) Data from Fig. 2a and b analyzed for dose-dependence of inhibition at a fixed concentration of lipid A (50 μg/ml). Data points represent the means ± standard errors of triplicate determinations.

coli O111:B4 S-LPS immobilized on microtiter plates. Control experiments with the 5B10 MAb have confirmed that these results are not due to an inability of *E. coli* O111:B4 S-LPS to absorb to the microtiter plates (data not shown).

The results summarized in the preceding paragraph suggested either that HA-1A does not bind to S-LPS or that the epitope on S-LPS recognized by HA-1A is masked when this antigen is absorbed to microtiter plates. To distinguish between these two possibilities, soluble antigen (S-LPS) competitive inhibition studies were carried out. Increasing concentrations of *E. coli* O111:B4 S-LPS were preincubated with a constant concentration of HA-1A at 23°C. After 2 h, the mixtures were centrifuged and the supernatants were titrated for residual binding to Re-LPS. The results of one such experiment are shown in Fig. 7 and confirm that S-LPS in solution can, in fact, compete for binding of HA-1A to Re-LPS. Also shown in Fig. 7, by way of comparison, are similar experiments carried out with lipid A and Re-LPS. While both of the latter lipid A-containing preparations yield approximately 50% inhibition with concentrations on the order of 3 μg/ml, almost 10-fold greater concentrations of S-LPS are required for equivalent inhibition.

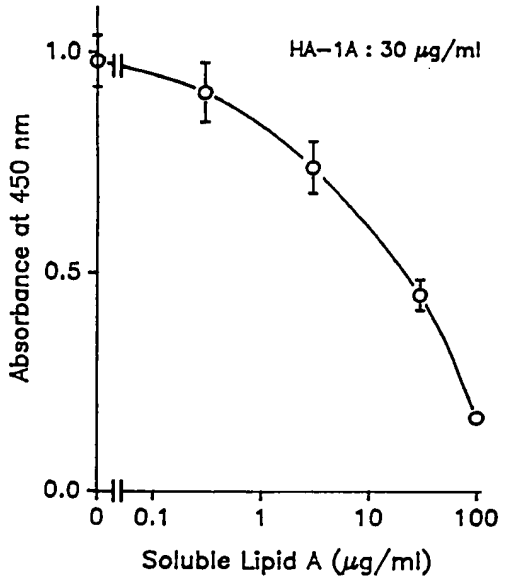

FIG. 4. Soluble antigen competitive inhibition assay by lipid A. Increasing amounts of lipid A were preincubated for 2 h at 23°C with 30 μg of HA-1A per ml and then were centrifuged for 10 min at 9,000 × *g*. Supernatants were assayed for residual binding to immobilized lipid A. Data points represent the means ± standard errors of triplicate determinations.

DISCUSSION

Our experiments have confirmed specific binding of IgM MAb HA-1A to isolated purified lipid A and to various lipid A-containing LPS preparations. Evidence to support this conclusion includes both direct binding studies as well as competitive inhibition assays with soluble antigen and a second antibody with known lipid A specificity. These data further suggest that the relative capacities of HA-1A to bind to several different R-chemotype LPS and lipid A preparations are similar, in contrast to those of the 8A1 anti-lipid A MAb whose binding is restricted to isolated lipid A and LPS from deep rough chemotype mutants.

It is intrinsically obvious that the specific binding of antibody to antigen would depend upon both the amount of available antigen and the antibody concentration. Furthermore, specific binding should be saturable under conditions of limited antigen concentration. We have been painstakingly careful in the development of both direct immunoassay conditions and competitive inhibition assays to ensure adherence to these fundamental binding parameters. Recently published reports which have questioned the capacity of HA-1A to bind specifically to lipid A or LPS (1, 2, 7) did not specifically address these variables and thus do not allow

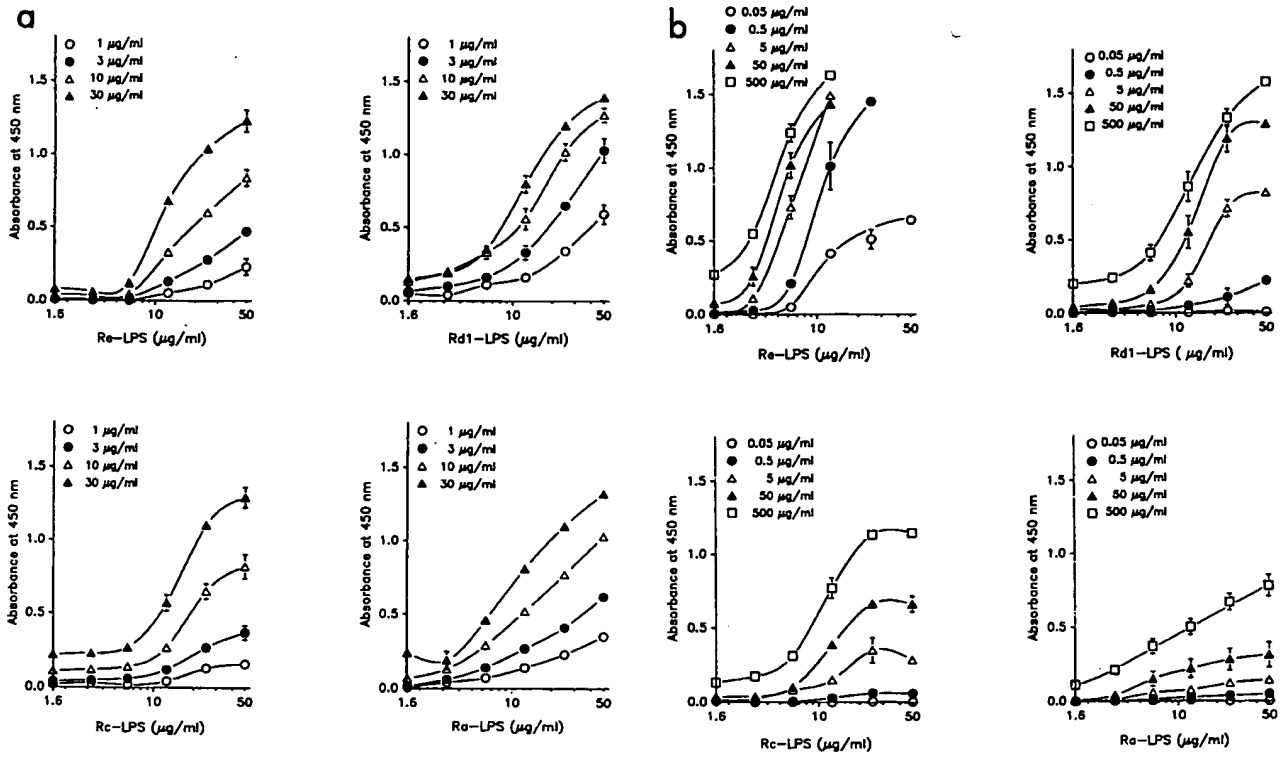

FIG. 5. Comparison of the binding of HA-1A (a) and 8A1 (b) to a panel of R-chemotype LPS. The 96-well microtiter plates were coated with twofold serial dilutions of Re-, Rd_1, Rc-, and Ra-LPS as described in Materials and Methods. HA-1A and 8A1 were diluted in PBS (pH 7.0) containing 0.1 M NaCl at 1, 3, 10, and 30 μg of HA-1A per ml and 0.05, 0.5, 5, 50, and 500 μg of 8A1 per ml and then were added to the plates.

valid conclusions to be drawn. Our own experimental design was based upon the concept proposed by Kuhn et al. (16), who suggested that, in ELISAs to detect lipid A specificity, "Titration of antigen with constant antibody dilution is a superior methodology to titration of antibody." However, to ensure that binding is dependent upon the concentration of the latter variable as well, we have varied both reactants in these experiments. We conclude from the results of the experiments reported here that, with respect to binding dependence upon antigen and antibody and saturability of binding, HA-1A does in fact bind specifically to lipid A and LPS.

This capacity of HA-1A to bind specifically to LPS and lipid A does not address the question of whether this antibody might bind to other antigens with structural similarity to lipid A. It is clear, however, that HA-1A does not bind to the majority of proteins contained in fetal calf serum, since this was used as an effective blocking agent in all of the ELISAs. In addition, we have shown that HA-1A does not bind to purified gangliosides (unpublished observations) or double-stranded DNA (11). It might not be totally unexpected to find at least some other antigens in nature to which HA-1A might bind.

Experiments carried out to compare HA-1A with the mouse IgG MAb 8A1 have provided some interesting insights into the lipid A-binding properties of MAb HA-1A. The 8A1 antibody has previously been demonstrated to bind with a high degree of affinity to lipid A (3), and those results have been confirmed in this study. Furthermore, we have shown that 8A1 will successfully compete with HA-1A binding to lipid A. While these results would be consistent with similarity in antigenic specificity of the two antibodies, they do not distinguish between antigenic identity, overlapping determinants, or steric inhibition. It is also possible that 8A1 may induce a conformational change in lipid A which prevents binding of HA-1A, and in this respect, we have not been able to demonstrate reciprocal competition inhibition of 8A1 binding by HA-1A (unpublished results). We have, however, recently shown that a murine IgM antibody with reported specificity for lipid A does not compete with HA-1A for binding to lipid A (11), supporting the concept that not all lipid A antibodies must, of necessity, manifest competitive inhibition.

It is also clear from these data that the relative affinity of 8A1 is significantly higher than that of HA-1A, as estimated either by the saturation binding profile (Fig. 2c and d) and the antibody competitive inhibition profile (Fig. 3c). Estimating a 50% inhibition at about 3 μg of 8A1 (an IgG) per ml for inhibition of 30 μg of HA-1A (an IgM) per ml, this suggests a relative effective binding affinity of HA-1A at about 1/50th that of 8A1. Because 8A1 has been reported to have a binding constant of $5 \times 10^9\ M^{-1}$ (3), an affinity of HA-1A of the order of $10^8\ M^{-1}$ might be anticipated, a value reasonably close to a recently reported experimental determination of binding affinity for this antibody (26).

Perhaps one of the most striking differences between HA-1A and 8A1 is the differential profile of these two antibodies in binding to R-chemotype LPS. Because both of these antibodies bind to lipid A, it is likely that the observed differential binding to R-chemotype LPS reflects intrinsic differences in epitope availability of lipid A within the macromolecular aggregate of the R-chemotype LPS. It is not

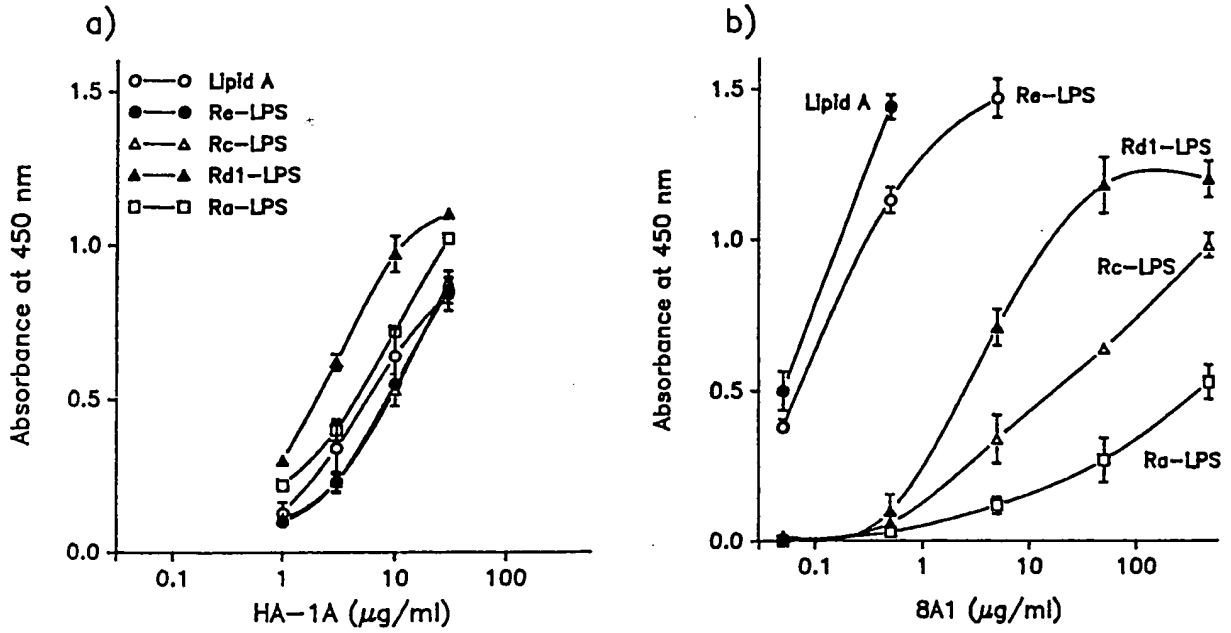

FIG. 6. Comparison of the dose-response curves of HA-1A (a) and 8A1 (b) to lipid A and a panel of R-chemotypes of LPS. The data presented are derived from results shown in Fig. 1 and 4 at an antigen concentration of 25 μg/ml. The background levels from the wells which were not coated with antigen (not shown in figures) were subtracted from each data point. Data points represent the means ± standard errors of triplicate determinations.

unreasonable to hypothesize, therefore, that higher-affinity antibodies might have more-precise requirements for epitope availability than those with lower relative affinity. In this respect, the potential availability of specific lipid A epitopes might well be influenced not only by aggregate LPS and/or lipid A structure but also by the presence of core oligosaccharide. The relative dependence of binding of anti-lipid A antibody to LPS chemotypes with increasing core oligosaccharide would be inversely proportional to effective binding affinity for lipid A. By this argument, the design of an MAb with optimal cross-reactive LPS binding properties would be one which maximizes broad spectrum binding without the deleterious consequences of high-affinity binding limitations.

It is of some interest that, while HA-1A does not bind to S-LPS on microtiter plates, it will, in fact, bind to S-LPS in solution. These results support the conclusion that S-LPS absorption to plastic may favor interactions of lipid A with plastic and that the high density of polysaccharide present in S-LPS sterically hinders HA-1A binding. It should be noted that our results contrast with those of Teng et al. (27) who originally reported that HA-1A would, in fact, bind to S-LPS via ELISA. The reasons for this are unknown but may, in part, reflect the different conditions used for the binding assays. These different findings underscore the earlier comprehensive studies by Pollack et al. (25) and Brade et al. (5) clearly documenting the importance of immunoassay design in the evaluation of antibody binding to LPS and lipid A antigens, particularly with respect to the physiochemical form of the lipid A-containing antigen (e.g., adsorbed to microtiter plates as soluble antigen, bound to erythrocytes, incorporated into liposomes), all of which can profoundly influence binding of anti-lipid A antibody. Our own studies showing that HA-1A will bind to S-LPS in solution but not to solid-phase S-LPS absorbed to microtiter plates is in accord with these conclusions. Furthermore, it is important to point out that we have noted considerable variability in the efficacy of various S-LPS to inhibit HA-1A binding to lipid A, even within different preparations of S-LPS from the same organism prepared by similar methods. The reasons for this are currently not known but nevertheless allow the conclusion that these results should not be extrapolated to mean that HA-1A will bind all S-LPS in solution.

It may be instructive to compare the different binding properties of the anti-lipid A antibodies described in this report with those of other MAbs or proteins known to bind lipid A. Within the framework of the former, it is interesting to note that a comprehensive study of anti LPS and lipid A MAbs by Pollack et al. (25) identified only one of 11 anti-lipid A IgM MAbs which demonstrated cross-reactive binding to R-chemotype LPS and select S-LPS. Similarly, we have recently provided preliminary data to suggest that mouse IgM MAb E5 also binds significantly less well to R-chemotype LPS than to lipid A (11). Thus, the capacity of HA-1A to bind to lipid A and R-chemotype LPS would appear not to be a common property of anti-lipid A MAbs.

Differential lipid A and R-chemotype LPS binding can also be demonstrated with nonantibody proteins. Two such serum proteins which have been previously demonstrated in our laboratory to bind LPS via interactions with lipid A are complement protein C1q and lysozyme. Of interest, the former protein is known to bind selectively to purified lipid A and Re-LPS and to manifest markedly reduced binding to Rc-LPS and S-LPS (8). This binding profile is precisely characteristic of the profile observed with MAb 8A1 (Fig. 6b). In contrast, lysozyme, which is also known to bind LPS via interactions with lipid A, appears to bind equally well to lipid A, all R-chemotypes, and S-LPS (22, 23). This binding profile is characteristic of the HA-1A binding profile. Thus, with these differential binding profiles for MAbs we find similar comparisons with nonantibody proteins and suggest

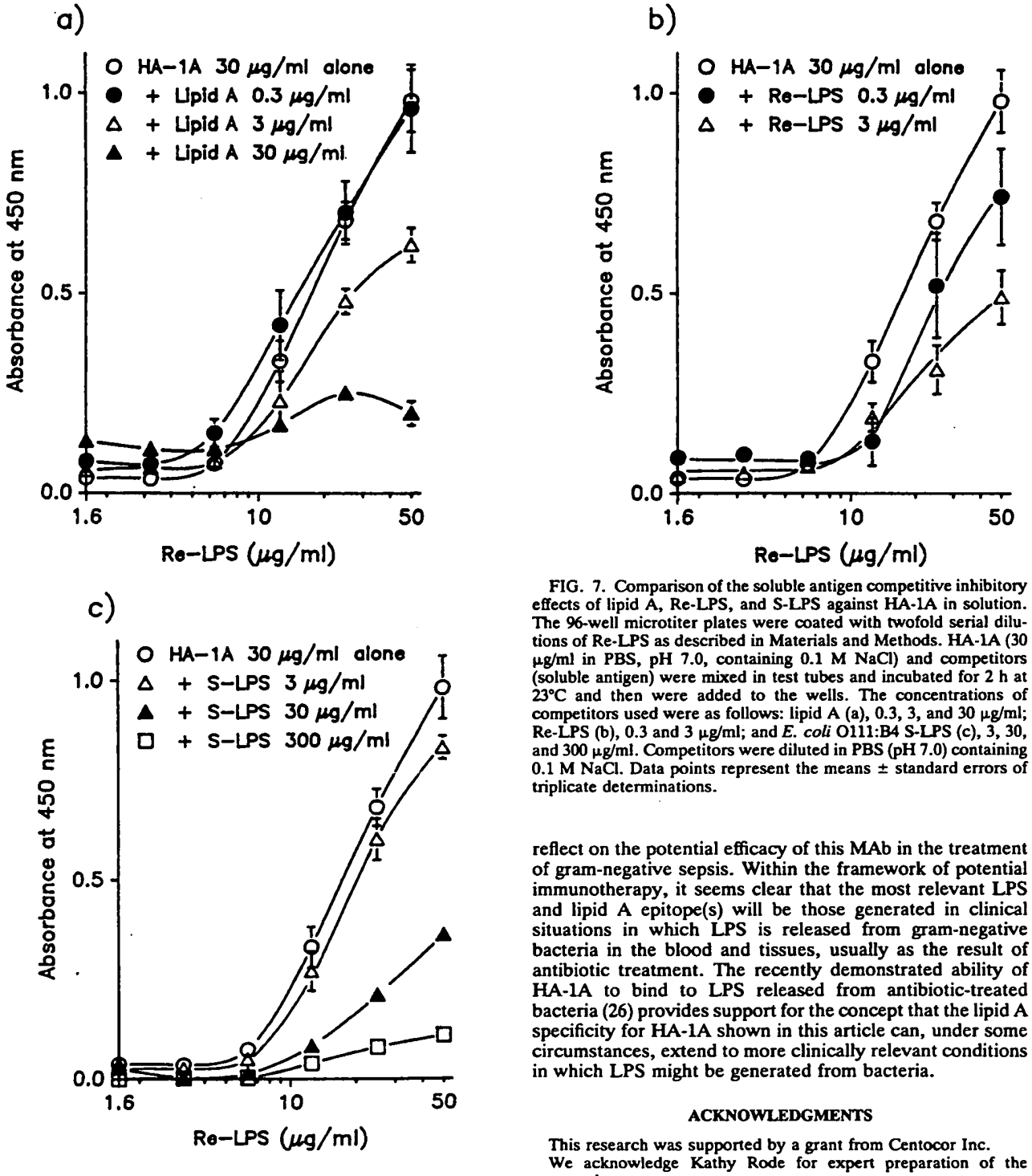

FIG. 7. Comparison of the soluble antigen competitive inhibitory effects of lipid A, Re-LPS, and S-LPS against HA-1A in solution. The 96-well microtiter plates were coated with twofold serial dilutions of Re-LPS as described in Materials and Methods. HA-1A (30 μg/ml in PBS, pH 7.0, containing 0.1 M NaCl) and competitors (soluble antigen) were mixed in test tubes and incubated for 2 h at 23°C and then were added to the wells. The concentrations of competitors used were as follows: lipid A (a), 0.3, 3, and 30 μg/ml; Re-LPS (b), 0.3 and 3 μg/ml; and *E. coli* O111:B4 S-LPS (c), 3, 30, and 300 μg/ml. Competitors were diluted in PBS (pH 7.0) containing 0.1 M NaCl. Data points represent the means ± standard errors of triplicate determinations.

that the observed differences between antibodies are the result of tertiary and quaternary configurations of the LPS macromolecular aggregate, which then define availability of lipid A-specific epitopes, rather than aberrant properties of the anti-lipid A MAbs per se.

Although the data in this article describe binding of HA-1A to purified LPS, these results do not necessarily reflect on the potential efficacy of this MAb in the treatment of gram-negative sepsis. Within the framework of potential immunotherapy, it seems clear that the most relevant LPS and lipid A epitope(s) will be those generated in clinical situations in which LPS is released from gram-negative bacteria in the blood and tissues, usually as the result of antibiotic treatment. The recently demonstrated ability of HA-1A to bind to LPS released from antibiotic-treated bacteria (26) provides support for the concept that the lipid A specificity for HA-1A shown in this article can, under some circumstances, extend to more clinically relevant conditions in which LPS might be generated from bacteria.

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